On the Regulation of the Serine Protease t-PA and its Inhibitor PAI-1 in the Brain

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Vi föddes
med en oerhörd saknad
genom ett
rusande tågfönster
om natten
ser du en skymt
av rymdens handstil
omöljlig att tyda
någonstans
i den slingrande väven
av gnistrande bläck
finns förklaringen
till våra liv

BRUNO K. ÖIJER
DIMMAN AV ALLT, 2001

ABSTRACT

The serine protease tissue-type plasminogen activator (t-PA) is the main fibrinolytic enzyme in the vascular system, and plays a critical role in the dissolution of thrombi. In recent years, researchers have focused on its role within the brain, where t-PA has been shown to participate in a number of physiological and pathophysiological processes, including various aspects of synaptic plasticity and neurodegeneration. To date, knowledge on how t-PA and its inhibitors are regulated in the brain has mainly been gained from murine *in vitro* and *in vivo* models. However, in view of the species-specific differences in the expression of these genes, the question remains as to how well the results obtained in animal models can be extrapolated to humans. Therefore, the work described in this thesis was directed at improving our knowledge on the regulation of t-PA and its principal inhibitor plasminogen activator inhibitor type-1 (PAI-1) in the human brain.

In this thesis, it is described for the first time that astrocytes have an intracellular storage compartment for t-PA, the levels of which can be increased in response to retinoic acid and protein kinase C activation. Regulated release of t-PA is induced in response to forskolin and histamine, which implies that astrocytes contribute to the extracellular levels of t-PA within the human brain. Expression studies of PAI-1 and of another t-PA inhibitor, protease nexin-1 (PN-1), revealed that the astrocytic expression levels of these inhibitors are regulated in a dynamic manner by injury-related factors, such as cytokines and hypoxia. This response may represent an important protective mechanism to reduce neurotoxicity under conditions of excessive t-PA activity, such as in the acute phase of cerebral ischaemia and in epilepsy. Given the compelling evidence that excessive t-PA activity results in the breakdown of the blood-brain barrier (BBB), the expression profiles of t-PA, PAI-1 and PN-1 were also investigated in a rodent *in vitro* model of the BBB. We report that the cocultivation of astrocytes and cerebrovascular endothelial cells potentiates astrocytic PAI-1 gene expression, and that this response is more pronounced in the presence of pro-inflammatory stimuli, e.g., lipopolysaccharide. These findings imply an important role for intercellular signalling between astrocytes and endothelial cells in the modulation of t-PA activity within the BBB.

As it has been shown that genetic variants, i.e. polymorphisms, at the t-PA and the PAI-1 loci affect the expression of these genes in endothelial cells, we investigated whether this is also the case in the brain. Allele-specific gene expression analyses revealed that polymorphisms located in the regulatory regions of the t-PA and PAI-1 genes affect their expression in human brain tissue and in human astrocytes, respectively. Furthermore, protein-DNA interaction studies demonstrated an altered binding of transcription factors to the polymorphic sites, which likely serves as the molecular genetic explanation behind these findings. Consequently, these polymorphisms could be used to explore the significance of differences in the expression levels of t-PA and PAI-1 in adequately powered clinical association studies.

Taken together, our findings elucidate the mechanisms through which t-PA and PAI-1 are regulated in the brain. This knowledge is expected to facilitate our understanding of how t-PA is involved in the processes of memory and learning, as well as in various neurological conditions associated with altered t-PA levels.

Key words: gene expression, astrocytes, blood-brain barrier, plasminogen activator inhibitor type-1, polymorphisms, protease nexin-1, tissue-type plasminogen activator, transcriptional regulation

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred in the text by their Roman Numerals:

I. Hultman K, Tjärnlund-Wolf A, Fish RJ, Wilhelmsson U, Rydenhag B, Pekny M, Kruithof EKO, Jern C. Retinoids and activation of PKC induce tissue-type plasminogen activator expression and storage in human astrocytes. *Journal of Thrombosis and Haemostasis* 2008;6:1796-1803

II. Hultman K, Blomstrand F, Nilsson M, Wilhelmsson U, Malmgren K, Pekny M, Kousted T, Jern C, Tjärnlund-Wolf A. Expression of plasminogen activator inhibitor-1 and protease nexin-1 in human astrocytes; response to injury-related factors. *Journal of Neuroscience Research* 2010;88:2441-2449

III. Hultman K, Björklund U, Hansson E, Jern C. Potentiating effect of endothelial cells on astrocytic plasminogen activator inhibitor type-1 gene expression in an *in vitro* model of the blood-brain barrier. *Neuroscience* 2010;166:408-415

IV. Hultman K, Tjärnlund-Wolf A, Odeberg J, Eriksson P, Jern C. Allele-specific transcription of the PAI-1 gene in human astrocytes. *Thrombosis and Haemostasis* 2010; August 30 [Epub ahead of print]

V. Tjärnlund-Wolf A, Hultman K, Curtis M, Faull RLM, Medcalf RL, Jern C. Allelic imbalance of tissue-type plasminogen activator (t-PA) gene expression in human brain tissue. *In manuscript*

CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
AP-1	activator protein-1
ATF	activating transcription factor
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CNV	copy number variation
CREB	CRE-binding protein
CV	coefficient of variation
DMEM	Dulbecco′s modified Eagle′s medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EGM-2	endothelial cell growth factor-containing medium-2
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FBS	foetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GFAP	glial fibrillary acidic protein
GRE	glucocorticoid-responsive element
haploChIP	haplotype-specific chromatin immunoprecipitation
HIF-1	hypoxia-inducible factor-1
HRE	hypoxia-responsive element
HUVEC	human umbilical vein endothelial cell
IL	interleukin
kb	kilobases
LD	linkage disequilibrium
LPS	lipopolysaccharide
LRP	low-density lipoprotein receptor-related protein
LTP	long-term potentiation
MEM	Eagle′s minimum essential medium
MMP	matrix metalloproteinase
mRNA	messenger RNA

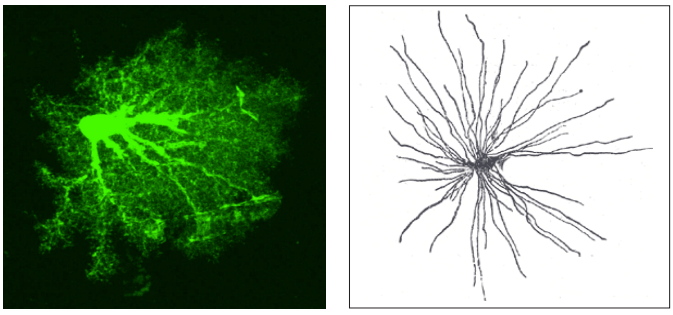
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PAI-1	plasminogen activator inhibitor type-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-CC	platelet-derived growth factor CC
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PN-1	protease nexin-1
qRT-PCR	quantitative reverse transcriptase Real-Time PCR
RA	retinoic acid
RAR	retinoic acid receptor
RARE	RA response element
RNA	ribonucleic acid
rt-PA	recombinant t-PA
SEM	standard error of the mean
SNP	single nucleotide polymorphism
Sp1	specificity protein 1
TGF	transforming growth factor
TNF-α	tumour necrosis factor-α
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
VLDLRE	very-low-density lipoprotein-responsive element

BACKGROUND

It has long been recognised that the serine protease tissue-type plasminogen activator (t-PA) is a key enzyme in the dissolution of blood clots within the vascular system. Consequently, the plasma level of t-PA has been implicated in cardiovascular diseases, including myocardial infarction and ischaemic stroke. However, in recent years, it has become apparent that the role for t-PA in the brain extends far beyond the regulation of intravascular fibrinolysis. In the central nervous system (CNS), t-PA is produced by virtually all cell types, whereas its main inhibitor, plasminogen activator inhibittor type-1 (PAI-1), is predominantly expressed by astrocytes. In the CNS, t-PA is implicated in a wide range of normal and pathophysiological processes, including various aspects of synaptic plasticity and neurodegeneration. Despite the vast amount of studies conducted in order to characterise the regulation of extracellular t-PA activity in various rodent *in vivo* and *in vitro* models, knowledge regarding its regulation in the human CNS remain scarce. Therefore, the overall aim of the present thesis was to increase insight into the mechanisms that regulate the expression of t-PA and its inhibitors in the human brain, with special emphasis on the role of astrocytes in modulating extracellular t-PA activity.

Astrocytes

The term astrocyte was introduced at the end of the 19th century, to refer to star-like or spider-like cells observed within the CNS. With the development of new staining and visualisation techniques, astrocytes are now defined as having a bushy appearance with a large number of fine processes that interact with cerebral blood vessels, neuronal synapses and other components of the brain parenchyma (Figure 1)[1]. In this context, it is noteworthy that a single astrocyte has the capacity to interact with tens of thousands or even up to a million synapses in the human brain[2]. Given these extensive cell-to-cell interactions, it is not surprising that astrocytes provide important structural, metabolic, and tropic support to neurons, both during embryonic development and in the adult CNS[3]. There is also evidence that astrocytes play an active role in the modulation of neuronal activity through the release of gliotransmitters, which occurs in response to changes in neuronal activity[4,5]. In addition to their role in normal neurotransmission, astrocytes are implicated in stroke, viral infection, epilepsy, and neurodegenerative diseases. In these pathological situations, astrocytes become activated through a process known as reactive gliosis, which is characterised by hypertrophy of the astrocytic processes and increased expression of intermediate filament proteins, including glial fibrillary acidic protein (GFAP), vimentin, and nestin[6,7]. Reactive astrocytes can migrate towards the injury and enclose the damaged tissue by forming a glial scar. Although this response may initially protect the noninjured tissue and preserve vital brain



A B

Figure 1. The astrocyte now and then. (A) Dye filling of an astrocyte reveals fine radial processes that give the cell a bushy appearance. Picture adapted from Wilhelmsson *et al*, 2004[12]. (B) Illustration of a spider-like astrocyte from the late 19th century. Source: Gray Henry. Anatomy of the Human Body. Philadelphia: Lea and Febiger, 1918; www.bartleby.com, 2000.

functions[8,9], the glial scar may subsequently hinder regenerative processes[10,11]. There is also a dynamic expression of pro- and anti-inflammatory cytokines in reactive astrocytes that may exert both beneficial and detrimental effects within the brain parenchyma[6].

The blood-brain barrier (BBB)

The BBB, which is a unique and specialised structure that separates the CNS from the vascular system, is formed by cerebrovascular endothelial cells that are surrounded by a basal lamina, pericytes, and perivascular astrocytic endfeet. These endothelial cells are connected by tight junctions and possess specific membrane transporter systems that selectively control the transport of molecules across the BBB[13]. The BBB is no longer viewed as an isolated barrier, but rather as a dynamic structure that is stringently regulated by environmental factors. Although the precise role of astrocytes in the BBB is still not well understood, evidence suggests that these cells are involved in the maintenance of the barrier[14]. Moreover, cerebral endothelial cells can influence the differentiation and maturation of astrocytes[15,16]. In addition to these long-term processes, there are cellular interactions that occur within seconds to minutes and involve receptor-mediated responses of endothelial cells, astrocytes and neurons. This intercellular signalling has been suggested to play a significant role in the con-

trol of local cerebral blood flow and energy supply to neurons[17]. This functional network of microvessels, astrocytes, and neurons has given rise to the concept of the neurovascular unit. Dysfunction of the neurovascular unit and loss of BBB integrity occurs in response to traumatic insults, cerebral ischaemia or inflammation[18]. This results in leakage of blood-borne substances and inflammatory cells into the brain parenchyma, which triggers the activation of astrocytes, resulting in both protective[19] and deleterious effects[20] on the BBB.

Tissue-type plasminogen activator (t-PA)

The human t-PA (*PLAT*) gene is localised to p12-q11.2 on chromosome 8 [21]. It consists of 14 exons that code for 527-530 amino acids of the mature protein, which has a molecular mass of ~70 kilodalton (kDa)[22]. The t-PA protein is a serine protease that is secreted in a single-chain form, which may subsequently be proteolytically converted into a two-chain form by plasmin. In contrast to most other serine proteases, the two forms of t-PA exhibit proteolytic activities[23]. Thus, interactions between t-PA and its inhibitors regulate the extracellular activity of t-PA. An additional plasminogen activator, urokinase-type plasminogen activator (u-PA), has also been identified. Whereas t-PA is best known for its role in the vascular system, u-PA is implicated in processes of tissue remodelling and cell migration, especially during cancer metastasis[24].

t-PA in the vascular system

The intravascular fibrinolytic system is regulated by numerous factors, the most important activator of which is t-PA. The endothelial cells that line the vascular wall can secrete t-PA into the vascular space, which in the presence of fibrin converts the proenzyme plasminogen to plasmin. The generated plasmin then cleaves fibrin and other components of the blood clot. Substances formed during the clotting process are potent triggers of the acute release of t-PA from the endothelium[25,26], and this acts as an important counter-regulatory mechanism to prevent the formation of occlusive thrombi. In line with its critical role in intravascular fibrinolysis, recombinant t-PA (rt-PA) is widely used as a thrombolytic therapy in ischaemic stroke and myocardial infarction. However, in ischaemic stroke, the use of rt-PA is limited due to the risk of bleeding, and therefore administration of rt-PA must be conducted within 4.5 hours of symptom onset in order to improve significantly the clinical outcome[27]. There is also considerable evidence to suggest that t-PA exert neurotoxic effects within the brain parenchyma, as discussed below. Therefore, much effort has been expended towards finding better thrombolytic alternatives. One agent that showed initial promise in experimental studies due to its lack of neurotoxic side-effects was Desmoteplase, a plasminogen activator that is derived from the saliva of the *Desmodus rotundus* vampire bat[28,29]. However, a Phase III clinical trail

(Desmoteplase In Acute Ischaemic Stroke-2, DIAS-2) was unable to detect any beneficial effects of Desmoteplase when given 3 - 9 hours after stroke onset[30]. A modest sample size, and the fact that only 30% of the included patients had a visible occlusion at presentation, may have contributed to the negative result of DIAS-2. Therefore, there are now two ongoing Phase III clinical trials on Desmoteplase in acute ischaemic stroke (DIAS-3 and DIAS-4).

t-PA in the CNS

Over the past few decades, it has become increasingly apparent that the action of t-PA is far more complex within the CNS than in the vascular system. The expression of t-PA is widely distributed throughout the brain parenchyma, and is most prominent in regions that are associated with a high degree of plasticity, including the thalamus, the amygdala, the hippocampus, and the cerebellum[31,32,33]. On the cellular level, neurons, microglia, astrocytes, Schwann cells, and cerebrovascular endothelial cells are potential sources of t-PA[31,32]. Several roles have been attributed to t-PA during embryonic development and in the adult brain. Two prominent areas in which t-PA is highly expressed during embryogenesis are the neural crest and the cerebellum[33], where t-PA facilitates cell migration[34,35]. The involvement of t-PA in neuronal cell migration is supported by the finding that t-PA can be axonally secreted and become bound to cell surface receptors, which concentrates t-PA at the growth cone[36]. Among the best-documented physiological functions of t-PA in the adult brain is its participation in synaptic changes associated with learning and memory formation. Experimental studies in mice have demonstrated that the t-PA gene is induced as an immediate-early gene within the hippocampus in response to stimuli that produce a long-lasting change in synaptic activity, i.e. long-term potentiation (LTP)[37]. Induction of t-PA is also elicited in the cerebellum after motor learning training[38], and in the amygdala after the learning of stress responses[39]. Studies in transgenic mice have revealed that the over-expression of t-PA results in an increased LTP response and improved performance in learning tasks[40], whereas deletion of the t-PA gene leads to a reduction in the LTP response and the extent of both hippocampal and cerebellar-dependent learning[41,42]. In line with this is the finding that exogenous t-PA enhances LTP, whereas inhibitors of t-PA reduce LTP[43].

Several mechanisms have been proposed to explain how t-PA modulates synaptic plasticity. One of the most extensively studied substrates for t-PA is plasminogen. Expression of plasminogen has been demonstrated in various regions of the brain, such as the cortex, the hippocampus, and the cerebellum[44]. The t-PA-mediated conversion of plasminogen to plasmin activates downstream proteolytic cascades, resulting in the degradation of extracellular

matrix (ECM) components and various cell-adhesion molecules[45,46], which is an essential process for neurite outgrowth and synaptic reorganisation. Plasmin also has the ability to activate factors that are potent modulators of synaptic plasticity, such as transforming growth factor β (TGF-β)[47], brain-derived neurotrophic factor (BDNF)[48,49], and nerve growth factor (NGF)[50]. Moreover, t-PA may influence plasticity independently of plasminogen, through direct interactions with cell surface receptors, including the *N*-methyl-D-aspartate (NMDA) receptor and the low-density lipoprotein receptor-related protein (LRP). NMDA receptors, which are glutamate-gated ion channels that are localised to excitatory synapses throughout the CNS, are classical memory and learning receptors. LRP is best known for its role in endocytosis, although in recent years it has also been shown to have important functions in cell signalling in various tissues, including the brain. Interaction of t-PA with either of these receptors on neuronal synapses can lead to increased intracellular Ca^{2+} levels and/or activation of the protein kinase A (PKA) signalling pathway[51,52], both of which events are critical for some forms of LTP[53,54]. It has also been suggested that t-PA modulates NMDA receptor signalling indirectly *via* its ability to signal through LRP[55,56].

In addition to the physiological effects of t-PA, it has become evident that t-PA acts as a potentiating factor in excitotoxicity, which is of particular relevance in the context of neuropathological conditions such as cerebral ischaemia and epilepsy. This is exemplified in studies of transgenic mice, in which t-PA deficiency protects against neuronal loss after intra-hippocampal injection of various glutamate agonists[57], and reduces the cortical injury that occurs after cerebral ischaemia and brain trauma[58,59]. Mice deficient for t-PA are also less susceptible to pharmacologically induced seizures[60]. The mechanisms underlying these effects of t-PA are complex and may include the potentiation of NMDA-mediated excitotoxicity[51,61], the triggering and activation of proteolytic cascades leading to degradation of the ECM[62,58], the activation of microglial cells[63,64], and opening of the BBB. The latter effect involves interaction of t-PA with LRP on perivascular astrocytes and the subsequent activation of platelet-derived growth factor CC (PDGF-CC)[65,66]. All of the above-mentioned events probably act in concert and result in exaggerated cell death, oedema, and haemorrhage. In addition to its involvement in neurotoxicity, there is evidence to indicate that t-PA may play a role in chronic neurodegenerative diseases, including Alzheimer's disease and multiple sclerosis. In these conditions, t-PA can have benifical effects by inducing degradation of amyloid-β and of fibrin deposits[67]. A summary of the possible physiological and pathophysiolgical effects of t-PA within the CNS is presented in Table 1.

Table 1.

EFFECT	POSSIBLE ROLE IN	
	PHYSIOLOGY	PATHOPHYSIOLOGY
Cleavage of plasminogen to plasmin	**SYNAPTIC PLASTICITY** ECM remodelling, activation of TGF-β, BDNF, and NGF	**EXCITOTOXICITY / OEDEMA** Degradation of ECM, activation of microglial cells
Interaction with NMDA receptor	**SYNAPTIC PLASTICITY** Enhanced LTP	**EXCITOTOXICITY** Overexcitation of neurons **SEIZURE SPREADING**
Interaction with LRP	**SYNAPTIC PLASTICITY** Potentiation of NMDA signalling	**EDEMA** Increased BBB permeability via activation of PDGF-CC

t-PA in the peripheral nervous system (PNS)

The presence of t-PA has been demonstrated in the PNS, both within axons that extend from neuronal cell bodies located within the CNS and in Schwann cells[68]. The expression of t-PA is upregulated in response to experimental nerve injury, which through t-PA-mediated degradation of fibrin may have beneficial effects on regenerative processes. This is evident in studies of transgenic mice, in which t-PA deficiency leads to exacerbated axonal degeneration[69] and delayed functional recovery[70] following peripheral nerve injury.

Serine protease inhibitors (serpins)

Serpins represents a large family of structurally related proteins that are characterised by their conserved mechanism of inhibition. These protease inhibitors contain a reactive centre loop that functions as a pseudosubstrate for the protease. After binding of the serpin to the protease, the reactive centre loop is cleaved by the protease. This results in major conformational changes, which ultimately render the protease inactive[71]. In the brain, the extracellular activity of t-PA is regulated by two major serpins: PAI-1 and neuroserpin. The serpin protease nexin-1 (PN-1) has also been shown to exert an inhibitory action on t-PA, although its role in this respect remains unknown.

PAI-1

The human PAI-1 (*SERPINE1*) gene is located at q21.3-q22 on chromosome 7. It consists of nine exons that code for 379 amino acids of the mature single-chain protein with a molecular mass of ~45 kDa[72], depending on its glycosylation pattern. PAI-1 is best known as the principal inhibitor of t-PA, although it also inactivates u-PA and has been implicated in various processes of cancer metastasis, including cell migration, adhesion, and angiogenesis[24].

In the vascular system, PAI-1 is synthesised and secreted in an active form, although it is unstable in solution and spontaneously converts into an inactive latent form[73,74]. The interaction between PAI-1 and t-PA occurs rapidly, with a second order rate constant of approximately 10^7 M^{-1} s^{-1}, and results in an irreversible complex[75,76]. While the cellular origin of circulating PAI-1 is not known, *in vitro* studies have demonstrated that PAI-1 is produced by various cell types, including endothelial cells, smooth muscle cells, macrophages, hepatocytes, adipocytes, and platelets[77]. Although approximately 90% of circulating PAI-1 is found in platelets, the majority of platelet PAI-1 is considered to be inactive[78]. Nevertheless, a recent study suggests that platelets constitute the major source of plasma PAI-1 in healthy individuals[79].

In the CNS, PAI-1 is predominantly expressed by astrocytes. Although PAI-1 is expressed at a low level under normal conditions[80,81], there is compelling evidence that local upregulation of PAI-1 occurs after cerebral ischaemia[81,82] and after neurotoxicity induced by various glutamate agonists[83]. This response may represent an important endogenous mechanism to reduce the deleterious effects of excessive extracellular activity of t-PA. Direct evidence to support this notion comes from experimental models of cerebral ischaemia, in which intraventricular infusion of PAI-1 results in reduced infarct size[84], whereas PAI-1-deficient mice display exacerbated brain damage[58]. Moreover, astrocytic expression of PAI-1, induced by TGF-β, has been shown to protect neurons against t-PA-mediated excitotoxicity *in vitro*[85]. In addition, PAI-1 may have anti-apoptotic effects that are independent of its inhibitory action[86].

Neuroserpin

Although neuroserpin was first identified as a serpin that is secreted exclusively from neurons[87,88], its expression has also been reported in cultured astrocytes[89]. In the extracellular space, neuroserpin forms inhibitory complexes with t-PA[90]. This interaction contrasts with that between t-PA and PAI-1, in that the t-PA-neuroserpin complex is relatively short-lived and after dissociation the activity of t-PA is fully restored[90]. Although this finding might argue for a less-important role for neuroserpin as a physiological regulator of t-PA, studies

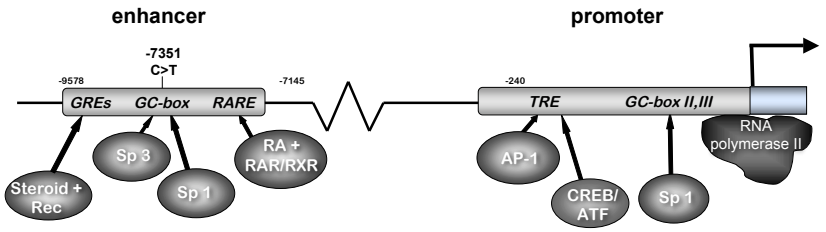


Figure 2. Schematic illustration of the regulatory regions within the t-PA enhancer and promoter.

of experimental cerebral ischaemia suggest that neuroserpin has neuroprotective effects that are related to its inhibition of t-PA[91,92,93].

PN-1

PN-1, which is also known as glia-derived nexin, is expressed by a variety of cells, including glial cells and neurons[94,95]. While the level of PN-1 expression is low under normal conditions, it is up-regulated following cerebral ischaemia, particularly in perivascular astrocytes[96,97]. This is in line with its main role as an inhibitor of the serine protease thrombin[98], which can enter the brain parenchyma during ischaemic stroke or after other insults that increase BBB permeability. In addition, at high concentrations, PN-1 forms complexes with t-PA, as well as with plasmin[98]. In support of its role as an inhibitor of t-PA, transgenic mice deficient in PN-1 show increased susceptibility to experimentally induced seizures[99]. However, the same study also provides counter-argument for the inhibitory action of PN-1 on t-PA, as the over-expression of PN-1 resulted in increased hippocampal LTP[99]. The physiological and pathophysiological effect of PN-1 on extracellular t-PA activity in the CNS thus remains to be elucidated.

Regulation of t-PA and PAI-1

The expression of protein-encoding genes is regulated at several different levels, particularly at the level of transcription. The initiation of gene transcription is a complex and finely tuned process, that is controlled by a multitude of sequence-specific regulatory proteins and transcription factors, which interact with the promoter and more distal elements, such as enhancers and repressors[100]. The term promoter refers to a cluster of elements located in proximity to

the transcriptional start site, and serving as the docking site for the factors that constitute the transcription initiation complex, including RNA polymerase II. Enhancers are control elements that increase transcription independently of their orientation, and they are often located several kilobases (kb) from the gene itself. Although the precise mechanism by which enhancers stimulate transcription over such long distances is not fully understood, it is generally accepted that these elements can recruit regulatory proteins that are involved in chromatin remodelling or stabilisation of the transcription initiation complex. Enhancers may also function as start sites for the formation of the transcription preinitiation complex, which directs RNA polymerase II to the transcriptional start site[101].

Transcriptional regulation

Given its central role in intravascular fibrinolysis, the transcriptional regulation of t-PA has primarily been investigated in endothelial cells. These studies have revealed that the t-PA gene contains several key regulatory regions located both within the promoter and at distant upstream locations. Initiation of t-PA transcription is mainly mediated by a TATA-less promoter[102], although a TATA-dependent initiation site has also been identified[22]. In the present thesis, positions in the t-PA gene are numbered relative to the TATA-less start site. The t-PA promoter contains several potential binding sites for the transcription factor specificity protein 1 (Sp1). Activation of two of these DNA motifs, the GC-box II (located at base pairs (bp) -71 to -65) and GC-box III (bp -48 to -42), has been shown to be essential for both constitutive and inducible transcription[103,104,105]. The t-PA promoter also contains a phorbol 12-myristate 13-acetate (PMA)-responsive element (TRE; bp -222 to -214)[104]. Induction of t-PA promoter activity by PMA occurs through direct activation of the PKC signalling pathway, which results in the binding of transcription factors belonging to the activator protein 1 (AP-1) and the CRE-binding protein (CREB)/activating transcription factor (ATF) families to the TRE site[103,106]. Given the potential roles of t-PA in memory and learning, it is of interest to note that CREB has been shown to be crucial for the formation of synaptic plasticity and LTP[107]. In addition, PMA has been reported to increase the binding of Sp1 to GC-box III, thereby facilitating t-PA gene transcription[103].

Studies conducted by Bulens and co-workers in the mid-1990s led to the identification of a multi-hormone-responsive enhancer 7 to 8 kb upstream of the t-PA transcriptional start site[108] (Figure 2). This DNA fragment is activated by all the classical steroid hormones, except estrogens [108], as well as by retinoids, such as retinoic acid (RA)[109]. The RA response element (RARE; bp -7319 to -7303) consists of two direct repeats of the GGGTCA motif with an inter-

vening spacer of 5 nucleotides (t-PADR5)[109], whereas the hormone-responsive unit compromises four glucocorticoid-responsive elements (GREs; bp -7501, bp -7703, bp -7942 and bp -7960, respectively)[108]. Experiments on transgenic mice have demonstrated important roles for both the promoter and enhancer in regulating t-PA gene expression in the developing and adult CNS[110,111].

Studies on the transcriptional regulation of the PAI-1 gene have revealed several regulatory elements within the PAI-1 promoter. These include three hypoxia-responsive elements (HREs; bp -195 to -188, bp -175 to -168, and bp -165 to -158)[112,113], a very-low-density lipoprotein-responsive element (VLDLRE; bp -672 to -657)[114], three TGF-β-inducible elements (between bp -730 and -280)[115], and a tumour necrosis factor-α (TNF-α)-responsive element located approximately 15 kb upstream of the transcriptional start site[116]. Accordingly, numerous growth factors, cytokines, and hormones influence PAI-1 gene transcription. Two of the most prominent stimulators are hypoxia (i.e. low oxygen tension) and TGF-β. The signalling pathways through which these factors induce transcription of the PAI-1 gene have been well characterised in various cell types[117]. Hypoxia activates several different pathways, ultimately resulting in binding of the transcription factor hypoxia-inducible factor-1 (HIF-1) to the promoter[113], whereas TGF-β-mediated PAI-1 gene transcription involves binding of the Smad3 and Smad4 proteins to the promoter[115].

It is noteworthy that the molecular genetic mechanisms involved in t-PA and PAI-1 gene regulation may be both cell type- and species-specific[118,119], thus underlining the complex transcriptional regulation of these genes. Intriguingly, gene expression studies of hippocampal brain tissues derived from various species, including humans, gerbils, rats, and mice, have revealed significant inter-species differences in the expression levels of t-PA[83]. This was an important reason for basing our studies on human cells rather than on cells of rodent origin.

Post-transcriptional regulation

In addition to transcriptional control, post-transcriptional regulation, reflecting the stability of the mRNA transcript and the efficiency with which it is translated, also contributes to the final protein levels. Studies of oocytes have demonstrated that cytoplasmic polyadenylation, i.e. elongation of the mRNA poly(A) tail, triggers the activation of t-PA mRNA translation[120]. A similar regulatory mechanism has been described for neurons, whereby t-PA mRNA transcripts are rapidly polyadenylated within the synaptic region after stimulation with glutamate, allowing for local regulation of t-PA translation[121]. Both cytokines and hormones, such as TGF-β and insulin, have been demonstrated

to modulate the stability of PAI-1 mRNA transcripts[122,123]. However, it remains unknown as to whether this makes a significant contribution to the regulation of PAI-1 expression.

Constitutive and regulated release

The mechanisms of t-PA secretion have been extensively studied in endothelial cells, and involve two different release pathways[124]. Once the newly translated t-PA protein is released from the endoplasmic reticulum, it is transported through the Golgi apparatus, where it is sorted into the regulated pathway or the constitutive pathway. In the regulated pathway, the protein is packed into storage vesicles, and released in response to extracellular stimuli, whereas in the constitutive pathway, the protein continuously exits the Golgi apparatus in transport vesicles that are destined to fuse with the cell membrane even in the absence of an extracellular stimulus[125]. In endothelial cells, t-PA is localised to the Weibel-Palade bodies[127], although other intracellular storage compartments have also been implicated[126], and is released in a regulated manner in response to various agonists, including stimuli that increase the intracellular concentrations of Ca^{2+} and cyclic adenosine monophosphate (cAMP)[127,128]. Studies of cultured neurons have demonstrated that high levels of t-PA are concentrated in dense core granules, which can be stored for longer time periods in dendritic spines prior to secretion induced by depolarising stimuli[129]. This allows for t-PA to be strategically positioned at the synapse and ready for immediate release in response to increased neuronal activity. Regulated release of t-PA has also been demonstrated in neuroendocrine cells, in which t-PA is stored in catecholamine-containing vesicles and released upon sympathoadrenal stimulation[125]. In contrast to t-PA, there is no evidence for regulated release of PAI-1 in brain-derived or endothelial cells.

Endocytosis

Endocytosis regulates the clearance of t-PA from the vascular and the extracellular space. In the vascular system, circulating t-PA, PAI-1 and t-PA/PAI-1 complexes are endocytosed *via* the LRP on liver parenchymal cells, and *via* the mannose receptor on liver endothelial cells[130,131,132]. LRP is also present on the cell surfaces of astrocytes, and mediates the clearance of t-PA from the extracellular space[61]. A similar mechanism has been described for neurons[52], although contradictory findings have been reported[61].

Genetic variations at the t-PA and PAI-1 loci

Genetic variations can be classified into two major groups: single nucleotide polymorphisms (SNPs); and structural variations (e.g. insertions/deletions or copy number variations [CNVs]). Although the majority of genetic varia-

tions within the human genome are non-functional, there is still a considerable amount of variations that alter the structure or the expression level of the encoded protein. The impact of such functional variations is dependent upon their relative positions in the DNA sequence. Thus, genetic variations situated in coding regions may cause changes in the amino acid sequence, whereas those located within non-coding regulatory regions may affect transcriptional activity, post-transcriptional processing or mRNA stability. Recent studies have demonstrated that genetic variations within regulatory regions account for a significant portion of inter-individual differences in human gene expression[133,134,135], and are associated with various human diseases[100].

Our research group has investigated mechanisms regulating endothelial t-PA release. In 1999, we showed that genetic variation at the t-PA locus is associated with vascular t-PA release rates *in vivo*[136], and this was later confirmed by another group[137]. To search for functional genetic variants, we re-sequenced the coding and regulatory regions of the t-PA gene, and identified several novel SNPs[138]. A single nucleotide transition (A to T) located within a GC-box at position -7351 in the t-PA enhancer showed the closest association to t-PA release rates[138]. Functional studies of the t-PA -7351C>T SNP demonstrated that the mutant T allele bound the transcription factors Sp1 and Sp3 with decreased affinities, which resulted in reduced transcriptional activity with this allele variant[139]. Additional studies on human umbilical vein endothelial cells (HUVECs) revealed that this SNP affected the expression of endogenous t-PA in response to various stimuli, acting through both the t-PA enhancer and through the proximal promoter[140]. These findings prompted us to design further experiments to test the hypothesis that the t-PA -7351C>T SNP affects t-PA gene transcription in human brain tissue.

Several polymorphisms have been identified at the PAI-1 locus. Particular attention has been focused on two common polymorphisms, a single nucleotide insertion/deletion (4G or 5G) polymorphism positioned 675 bp upstream of the PAI-1 transcriptional start site, and a single nucleotide transition (A to G) polymorphism further upstream at -844 bp. These two promoter polymorphisms are in strong linkage disequilibrium (LD)[141,142], and have been associated with altered plasma levels of PAI-1 and with myocardial infarction[143,144,145,146], although conflicting findings have been reported[141,142,147,148]. Experimental studies of the PAI-1 -675(4G/5G) polymorphism have suggested that this polymorphism is functional at the level of transcription[114,149,150]. Transient transfections of HUVECs and HepG2 cells conducted by Eriksson and co-workers showed that cells that harboured the 5G allele variant exhibited reduced transcriptional activity, as compared to cells that were transfected

with the 4G allele variant[150]. A clue to the molecular mechanisms behind this response is the finding that both the 4G and 5G alleles contain a binding site for a transcriptional activator, whereas the 5G allele also binds a transcriptional repressor protein to an overlapping binding site[149,150]. However, other *in vitro* studies using various cell types have not provided unambiguous evidence as to whether this polymorphism affects basal and/or stimulated PAI-1 gene expression[146,149,151,152]. Intriguingly, the 4G high-expressing allele appears to have a protective effect in ischaemic stroke[153,154]. Although the mechanism underlying this effect remains unknown, one possible explanation is that high-level expression of PAI-1 in astrocytes reduces the neurotoxic side-effects of t-PA, thereby improving stroke outcome. With these studies in mind, we sought to investigate whether the PAI-1 -675(4G/5G) polymorphism affects PAI-1 gene transcription in human astrocytes.

AIMS OF THE THESIS

The work described in this thesis is directed at improving our knowledge on the regulation of t-PA and its inhibitors in the human brain.

The specific aims of this thesis were to:

- test the hypothesis that t-PA gene expression in human astrocytes is regulated by RA and the PKC activator PMA, and that t-PA is stored and is subjected to regulated release from these cells

- characterise the effects of injury-related factors on the expression of PAI-1, PN-1 and neuroserpin in human astrocytes

- test in an *in vitro* model of the BBB the hypothesis that cerebrovascular endothelial cells influence the gene expression of t-PA, PAI-1, and PN-1 in astrocytes

- examine whether the PAI-1 -675(4G/5G) promoter polymorphism affects the transcriptional activity of the PAI-1 gene in human astrocytes

- assess the effect of the t-PA -7351C>T enhancer SNP on t-PA gene expression in human brain tissue

METHODS AND METHODOLOGICAL CONSIDERATIONS

While the materials and methods used in this work are thoroughly described in the papers at the end of this thesis, more general descriptions and explanatory comments are presented below.

Human cell culturing and brain tissue

Human astrocytes

In Papers I, II, IV, and V, cell culture experiments were performed using human astrocytes (Clonetics, Walkersville, MD, USA; and ScienCell, San Diego, CA, USA). Cryopreserved astrocytes, which were harvested from the brains of foetuses of gestation age 17.5 to 23 weeks, were cultured in astrocyte growth medium (AGM, Clonetics or ScienCell) that was supplemented with 3% foetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. The medium was replaced every 2-3 days. Astrocyte cultures were >95% GFAP-positive, and were cultured for a maximum of six passages.

Human umbilical vein endothelial cells (HUVECs)

HUVECs were used for the immunocytochemical analyses (Paper I), and for preparation of nuclear extracts used in the electrophoretic mobility shift assay (EMSA) (Papers IV and V). Cells were derived from fresh umbilical cords obtained from the maternity ward at the Department of Obstetrics, Sahlgrenska University Hospital/Östra, Gothenburg, and isolated using a modified sterile technique[155]. Cells were grown at 37°C in a 5% CO_2 atmosphere in endothelial cell growth factor-containing medium-2 ((EGM-2, Clonetics) that was supplemented with 2% FBS. The medium was replaced every 2-3 days, and cells were cultured for a maximum of three passages.

> *Comments: In vitro* cell culturing is an invaluable experimental tool for studying cellular properties and functions. However, this system also has the inherent disadvantages that the cells are cultivated in an artificial milieu, which fails to mimic accurately the natural extracellular environment. Therefore, extrapolations of the results of *in vitro* experiments to the *in vivo* situation should be made with caution.

Human brain tissue

In Papers I and II, immunohistochemistry was performed on brain tissues that were resected from patients undergoing surgery for epilepsy at the Sahlgrenska University Hospital. Analysis of allele-specific t-PA gene expression (Paper V) was carried out on post-mortem brain tissues obtained from the Neurological

Foundation of New Zealand Human Brain Bank.

In vitro model of the blood-brain barrier (BBB)

In Paper III, cell culture experiments were performed using an *in vitro* model of the BBB. Preparation and culturing of cells was performed as described previously[156]. In brief, primary astroglial cultures were prepared from newborn rat cerebral cortices and cultured in Eagle´s minimum essential medium (MEM, Invitrogen, Paisley, UK), that was supplemented with 20% foetal calf serum, 7.5 mM glucose, 2× amino acids, 4× vitamins, 52.4 mM $NaHCO_3$, 2 mM L-glutamine, 1% penicillin (Invitrogen), and 0.5% streptomycin (Invitrogen). After 6 days in culture, astroglial cells were cocultured with newly prepared microvascular endothelial cells grown on Transwell permeable inserts (Corning Costar, Cambridge, MA, USA that were placed above the astrocyte cultures. The endothelial cells were isolated from brain cerebral capillaries and cultured as described previously[156], using the modification of Abbott and co-workers[157]. The endothelial cell culture medium comprised Dulbecco´s modified Eagle´s minimum essential medium (DMEM, low-glucose [1000 mg/l]; Invitrogen) that contained 20% horse serum (ICN Biomedicals GmbH, Meckenheim, Germany), 50 µg/ml gentamycin, and 2 mM L-glutamine. Monocultures and cocultures were grown at 37°C in a 5% CO^2 atmosphere, and the medium was replaced every 2-3 days. Astrocytes and endothelial cells were cocultured for 9-11 days prior to use in experiments.

Comments: Studying the BBB *in vivo* is difficult given the complexity of its structure. Most of the data collected to date comes from various *in vitro* models of the BBB[158]. The model utilised in the present thesis consists of a two-chamber system with monolayers of primary astrocytes and cerebrovascular endothelial cells, which are co-cultured on opposite sides of a synthetic permeable membrane. Although the two cell types in this system are never in contact, the Transwell membrane permits the exchange of soluble substances between the different cell compartments. Thus, this model has the advantage in that it permits for an easy collection of the two different cell types for subsequent anayses. A major limitation of this *in vitro* model is that other cell types within the neurovascular unit, such as pericytes and neurons, are not present. Moreover, the astrocytes and endothelial cells are not in direct contact with each other, in contrast to the *in vivo* situation. An illustration of the BBB model used in the present thesis is depicted in Figure 3.

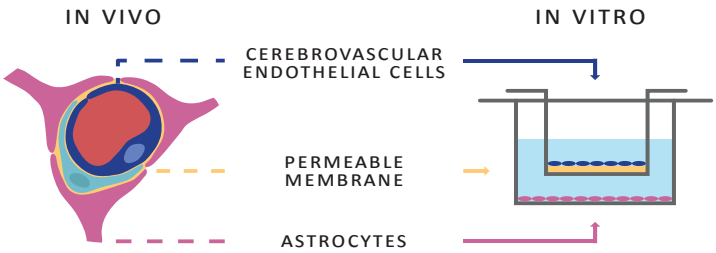


Figure 3. Schematic illustration of the two-chamber BBB model utilized in the present thesis.

Immunohistochemistry and cytochemistry

In Papers I and II, localisation patterns of t-PA and PAI-1 in human brain tissues were analysed using immunohistochemistry. The tissues were fixed in 4% paraformaldehyde, and immersed in 30% sucrose. Free-floating sections were pre-incubated in phosphate-buffered saline (PBS) that contained 0.5% Triton-X and 1% bovine serum albumin (BSA), so as to permeabilise the tissue and reduce non-specific binding of antibodies. The sections were incubated with primary antibodies, followed by the addition of appropriate Alexa Fluor-conjugated secondary antibodies (as listed in Papers I and II). Astrocyte cell bodies were identified by staining for the astrocyte-specific marker GFAP. Nuclei were visualised by adding TOPRO-3 to the final incubation step. Sections were mounted on slides and examined under a laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany).

Immunocytochemistry was used to study the intracellular localisation of t-PA in cultured human astrocytes and HUVECs (Paper I). Cells were cultured on glass coverslips, fixed with 4% paraformaldehyde, and pre-incubated with TBS that contained 0.1% Triton-X and 3% donkey serum. Coverslips were then incubated with mouse anti-human t-PA antibody, and then with an Alexa Fluor 555-conjugated donkey anti-mouse antibody. Nuclei were visualised by adding the Hoechst 33258 reagent to the final incubation step. Slides were mounted and examined under a fluorescent microscope (Nikon Eclipse E600; Nikon Corporation, Kanagawa, Japan).

> *Comments:* Immunohistochemistry and cytochemistry are sensitive methods for the localisation of antigens in tissue sections or cultured cells. Although the techniques used are fairly simple, the outcomes are influenced by several factors, such as the fixation and permeabilisation of the cells, sampling and processing of tissue samples, and the specificities of the primary antibodies used. Careful selection of the primary antibodies is crucial, as non-specific binding leads in false-positive results.

In the present thesis, to control for non-specific binding of secondary antibodies, sections incubated without the addition of primary antibody were included in all experiments.

Quantitative reverse transcriptase Real-Time PCR (qRT-PCR)

Analyses of mRNA expression levels in cultured cells (Papers I–III) were performed using qRT-PCR. Cells were harvested, and total cellular RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA was converted to complementary DNA (cDNA) by reverse transcription (GeneAmp RNA PCR kit; Applied Biosystems, Foster City, CA, USA). Target mRNA was quantified as described previously[139,159], with the modification that the PCR was performed in a 384-well format in an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) in a total volume of 10 µl. For amplification of the genes of interest, primers and probes were purchased directly from Applied Biosystems (listed in Papers I–III). The value for each sample was normalised to the expression level of an endogenous control gene, i.e. glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S. The relative quantification of gene expression was achieved by calculating the treatment-to-control expression ratio using the comparative C_T method. Each sample was analysed in triplicate for both the target gene and control gene.

Comments: qRT-PCR is a sensitive method used for the quantification of specific mRNA transcripts within a sample. Real-time detection has advantages over traditional PCR, in that the kinetics of the reaction is measured during each cycle and that data collection in the linear phase of the PCR is possible. The quantification of mRNA transcripts can be either relative or absolute. Relative quantification involve comparing the expression levels of a target gene to a reference gene that is constitutively expressed, thereby providing an internal standard to control for variabilities in RNA loading and the efficiency of the qRT-PCR. In preliminary experiments for this thesis, the expression levels of several reference genes were evaluated for their responses to various stimuli using an endogenous control assay (TaqMan Human Endogenous Control Plate; Applied Biosystems). Based on these results, the reference genes were selected.

Enzyme-linked immunosorbent assay (ELISA)

The constitutive release of t-PA and PAI-1 protein levels in the cell culture medium was determined using ELISAs (TintElize®t-PA and TintElize®PAI, respectively; Biopool® International, Umeå, Sweden) (Papers I and II). The mean intra-assay coefficients of variation (CVs) were 2.5% and 2.7% for t-PA and PAI-1, respectively. For studies of regulated release of t-PA from human

astrocytes (Paper I), a sensitive ELISA was used[127], the mean intra-assay CV of which was 8.1%. All samples were assayed in duplicate.

Comments: The t-PA and PAI-1 ELISAs used in the present thesis detect all molecular forms of the respective protein, i.e. both the active and the complex-bound forms. When investigating constitutive and regulated release, this is the method of choice because the total amount of protein has to be determined. From a biological point of view, it may also be of interest to determine the amount of active t-PA in the cell culture medium. However, in view of the very high kinetics of inhibition of t-PA by PAI-1, and the excess of PAI-1 over t-PA in astrocyte conditioned medium, most of the t-PA is complexed to PAI-1 which results in a major underestimation of t-PA activity *in vitro*. Analysis of t-PA activity was therefore not performed on astrocyte cell culture medium.

Electrophoretic mobility shift assay (EMSA)

In Papers IV and V, EMSA was employed to detect sequence-specific DNA-protein interactions. The preparation of nuclear extracts from human astrocytes, HUVECs, and neuronal-like NT2 cells was performed either as previously described[160], or using a nuclear/cytosol fractionation kit (BioVision Inc, Mountain View, CA, USA). Labelling of HPLC-purified oligomers, annealing, oligomer processing and preparation for EMSAs were performed as previously described[139]. Binding reactions were carried out with nuclear extracts in Osborne buffer D and SMK buffer that contained poly[d(I-C)]. The ^{32}P-labelled probe was added and the mixture was electrophoresed on a 5% polyacrylamide gel, and visualised in a phosphoimager (FLA-2000; Fuji, Stamford, CT, USA). To identify the specific proteins involved in DNA binding, supershift experiments were performed using antibodies directed against selected transcription factors (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Comments: EMSA is an important *in vitro* technique for assessing the sequence-specific DNA-binding of proteins and transcription factors. As radioisotope-labelled probes are used in the detection system, this technique is extremely sensitive. The major limitations of EMSA is that the length of the probe is restricted to approximately 20-30 bp, and thus the DNA-protein interactions are studied in the absence of the *in vivo* chromatin structure. Control experiments using various concentrations of competitors are vital to verify protein-binding specificity. This has been done in the present thesis; all the EMSAs included both specific and non-specific competitors. The principle of EMSA is illustrated in Figure 4.

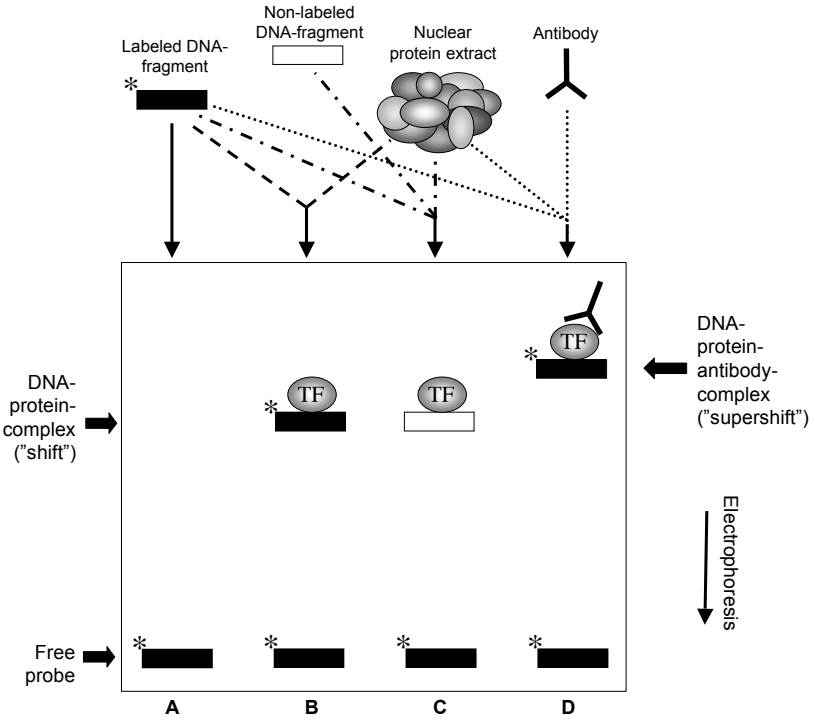


Figure 4. Principle of EMSA. In the EMSA, a ^{32}P-labelled DNA fragment (i.e., a probe) that contains a specific DNA site is incubated with a purified protein or crude nuclear extracts. The DNA-protein complexes are separated from unbound DNA by electrophoresis through a nondenaturing polyacrylamide gel. Probes with bound proteins (e.g., transcription factors; TF) migrate more slowly than an unbound probe, generating a "shift", which can be visualised by exposure of the gel to an X-ray film or a phosphoimager (A and B). Protein binding specificity can be determined by the addition of excess amounts of unlabeled competitors, which can be a specific probe with identical sequence to the radiolabelled probe (specific competition, C), or a non-specific probe that does not compete for specific binding of proteins to the radiolabelled probe (not shown). The identification of a protein that is bound to the labelled probe can be accomplished by including an antibody directed against a candidate protein. If the protein of interest is present, the antibody will bind to the specific protein-probe complex, thereby further decreasing its mobility, and resulting in shifting of the complex (termed supershifting, D).

Chromatin immunoprecipitation (ChIP)

ChIP was used to investigate potential interactions between the transcription factor Sp1 and the t-PA enhancer and promoter regions (Paper V). In cultured cells, chromatin was cross-linked by the addition of 1% formaldehyde, and cells were washed with PBS that contained proteinase inhibitors. To generate shorter DNA fragments, chromatin was sonicated using a VibraCell Sonicator (Chemical Instrument AB, Sollentuna, Sweden), and ChIP was performed according to the ChIP assay protocol (Upstate Biotechnology, Charlottesville, VA, USA). In brief, protein A-agarose beads were incubated with antibodies directed against Sp1 or IgG (Upstate Biotechnology). The agarose bead-antibody complexes were then added to chromatin. After washing to remove unbound antibodies, the samples were treated with RNase A and proteinase K, to reverse the cross-linking. The precipitated DNA fragments were resuspended in TE buffer and DNA was purified by ethanol precipitation. Two fragments, encompassing the Sp1 binding sites in the t-PA enhancer and promoter regions, were amplified using PCR (primer pairs listed in Paper V). The PCR products were visualised by separation on a 2% TAE-agarose gel that contained ethidium bromide.

> *Comments:* ChIP is used to study the interactions between transcription factors and specific DNA sequences under *in vivo*-like conditions, e.g., in cells that harbour an intact chromatin structure. Among the most important factors for a successful ChIP is the protein binding affinity of the antibody. This is crucial for the recovery of DNA fragments in the precipitation step. In contrast to EMSA, the ChIP technique does not allow for the identification of the specific DNA sequences involved in the DNA-protein interactions.

Relative allele-specific mRNA expression analysis

In Paper V, analysis of allele-specific mRNA expression of the t-PA gene was performed in human post-mortem brain tissues using a quantitative TaqMan genotyping assay. This approach requires for the SNP to be present in the RNA transcript, or that it is in strong LD with a coding SNP in the gene of interest. Therefore, the coding t-PA 20099T>C SNP, which is strong LD with the t-PA -7351C>T SNP, was used as a marker.

DNA extraction and genotyping

To identify samples that were heterozygous for the t-PA -7351C>T SNP and the t-PA 20099T>C SNP, genomic DNA (gDNA) was isolated from human brain tissue samples using an E.Z.N.A Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. Genotyping for both SNPs were performed by TaqMan-based allelic discrimination assay (Applied Biosystems)161, and by sequencing (assays and primer pairs listed in Paper V).

Quantitative TaqMan genotyping assay

Differences in allele-specific t-PA mRNA expression were analysed using the TaqMan assay used for genotyping of the t-PA 20099T>C SNP, with the modification that the reaction was recorded in real-time on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) and involved either 10 ng of gDNA or 10 ng of cDNA. To correct for subtle differences in the amplification rates of the two alleles, a standard curve for each fluorophore was created by mixing gDNA from two homozygous individuals in different allelic ratios. The cDNA and gDNA allelic ratios for each heterozygous sample were then extrapolated from the standard curves. Assuming that the two alleles are present in equal amounts in gDNA[162], the cDNA ratios were adjusted for the mean gDNA allelic ratio. Each heterozygote cDNA sample and its corresponding gDNA sample were assayed in triplicate, and each experiment was repeated on two separate occasions.

Comments: The effect of regulatory SNPs on transcriptional activity can be analysed in cDNA samples from heterozygous individuals using various genotyping techniques, such as quantitative TaqMan genotyping assay. These techniques are superior to traditional reporter vector assays, in that the two alleles are analysed within the same sample rather than between samples, thus avoiding the effects of variations in environmental factors, such as tissue sampling and RNA quality. In contrast to the t-PA -7351C>T SNP, the PAI-1 -675(4G/5G) polymorphism is not in LD with any coding SNP. Thus, for the relative allele-specific expression analysis of PAI-1 a more novel approach had to be adopted, as described below.

Haplotype-specific chromatin immunoprecipitation (haploChIP)

The haploChIP approach was employed in Paper IV, to quantify the allele-specific promoter activity of the PAI-1 gene in human astrocytes.

To identify heterozygous astrocytes, cells from different individuals were cultured as described above and genotyped for the PAI-1 -675(4G/5G) polymorphism and the PAI-1 -844A/G SNP using TaqMan genotyping assay (Applied Biosystems) and sequencing (assays and primer pairs listed in Paper IV). The haploChIP assay was performed essentially as described above for ChIP, with the exception that haploChIP required a higher concentration of starting material (approximately 5×10^8 cells per IP) and was performed using a ChIP-IT kit (Active Motif, Rixensart, Belgium). Immunoprecipitations were performed using antibodies directed against total RNA polymerase II (Active Motif, Carlsbad, CA, USA), antibodies directed against active RNA polymerase II (Covance Laboratories, Richmond, CA, USA) or IgG (Upstate Biotechnology).

Nested-PCR, which was used to amplify a fragment that encompasses the PAI-1 -675(4G/5G) polymorphism, was performed as described in Paper IV. After PCR amplification, the product was prepared for pyrosequencing according to the manufacturer's protocol (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing was performed using a Pyrosequencing Reagent kit (Pyro Gold, Biotage, Uppsala, Sweden). In brief, the biotinylated PCR product was immobilised on streptavidin-coated sepharose beads, aspirated, and washed with 70% ethanol, denaturing buffer and wash buffer. Subsequently, the beads were released into pyrosequencing reaction plates that contained annealing buffer and the sequencing primer (listed in Paper IV). Primer annealing was performed by heating the samples to 80°C for 2 min. The pyrosequencing data were quantified using the PSQ 96MA system (Pyrosequencing AB). Each sample was analysed in duplicate on two separate occasions. Schematic illustrations of the haploChIP approach and pyrosequencing of the PAI-1 -675(4G/5G) polymorphism are depicted in Figure 5 and 6, respectively.

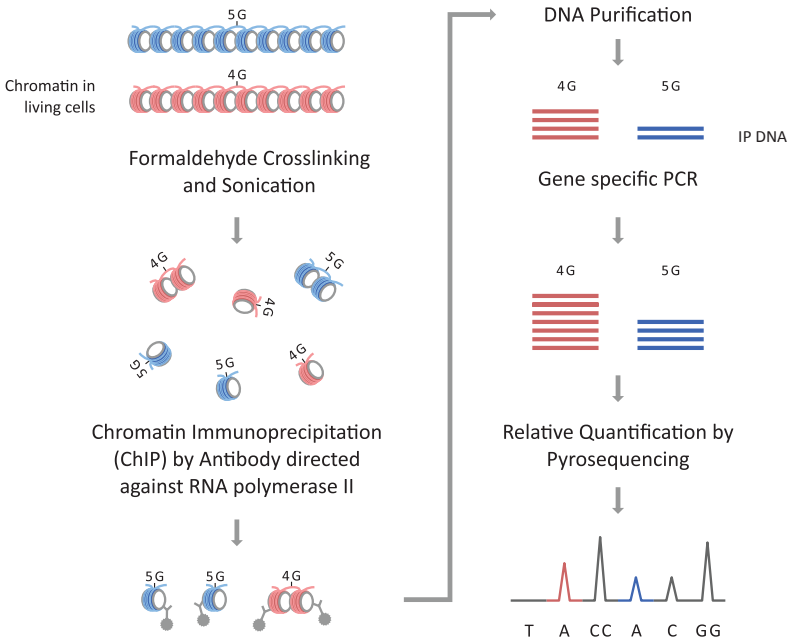


Figure 5: HaploChIP of the PAI-1 -675(4G/5G) polymorphism. In heterozygous living astrocytes, DNA and bound proteins are cross-linked with formaldehyde, and the DNA is fragmented by sonication (A). Antibodies directed against RNA polymerase II are used to immunoprecipitate those DNA fragments that are actively undergoing transcription (B). The cross-links are reversed, and the purified DNA is amplified by gene-specific PCR (C). The relative abundance of each allele in the amplified DNA is subsequently determined by quantitative allele-specific analysis (D), as described in Figure 6.

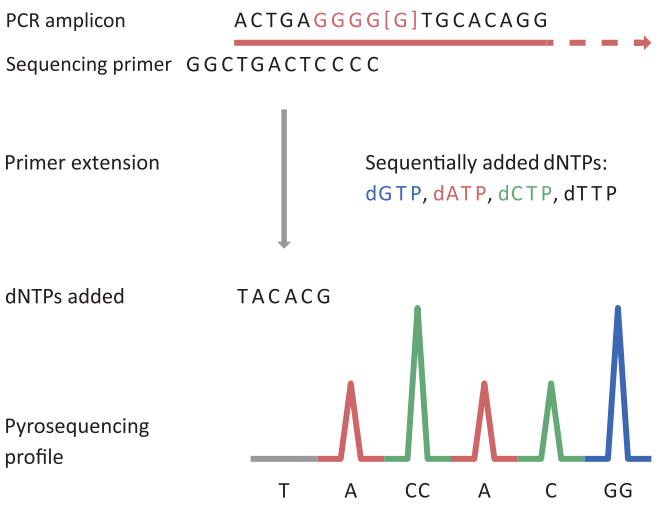


Figure 6: Pyrosequencing of the PAI-1 -675(4G/5G) polymorphism. The sequencing primer is hybridized to the PCR amplicon. Deoxyribonucleotide triphosphates (dNTPs) are sequentially added, and when incorporated into the DNA strand, ATP is generated. This ATP drives an enzymatic reaction that results in the emission of light, the amount of which is proportional to the amount of generated ATP. The emitted light is detected by a camera and visualised as a peak in the pyrosequencing profile, the height of which corresponds to the number of nucleotides incorporated.

Comments: The haploChIP approach is based on ChIP of phosphorylated RNA polymerase II followed by relative quantification of the precipitated DNA molecules[163]. Differences in phosphorylated RNA polymerase II loading between two alleles in a heterozygous sample provide a measure of allele-specific transcriptional activity. As the haploChIP approach is based on the ChIP methodology, it allows for allele-specific expression studies within the intact chromatin structure. A limitation of this approach is that it requires that the polymorphism is located within 1 kb of the transcriptional start site. For this reason, we were not able to utilise this method for functional analysis of the t-PA enhancer -7351C>T SNP.

Statistical analysis

Values are presented as mean and standard error of the mean (SEM). A *P*-value equal to or less than 0.05 was considered statistically significant. In Papers I and II, time-dependent changes in the mRNA and protein levels in response to treatment were evaluated by one-way analysis of variance (ANOVA). When ANOVA indicated a significant overall effect, post-hoc analysis was performed with Tukey's test. An independent sample *t*-test was used to evaluate differences in responses to treatment between different groups (Papers I and III). In Paper V, differences in allelic expression ratios were tested using a one-sample *t*-test, and correlation analysis was performed with Spearman's test.

RESULTS AND DISCUSSION

Induction, storage, and regulated release of t-PA from human astrocytes (Paper I)

In contrast to endothelial cells, little is known about the regulation of t-PA gene expression in astrocytes. In Paper I, we investigated whether t-PA gene expression in human astrocytes is regulated by RA and the PKC activator PMA, and examined whether t-PA is stored and subject to regulated release from these cells, as previously demonstrated for t-PA in endothelial cells.

Following treatment with RA or PMA, we observed a pronounced upregulation of t-PA mRNA and protein expression, with an approximately 4-fold induction after prolonged stimulation (20 hours). This response is of similar magnitude to that previously reported in endothelial cells[164,165,166]. As endothelial cells store t-PA intracellularly[127,126], we next examined if this was also true for astrocytes. Using immunocytochemistry, we identified an intracellular compartment for t-PA in astrocytes. Induction of t-PA by RA and PMA resulted in an increased storage and proportion of t-PA-expressing cells. The distribution of t-PA-positive granules in astrocytes was similar to that observed in endothelial cells, with an accumulation in the perinuclear area[127,126]. These novel findings led us to investigate whether t-PA undergoes regulated release from astrocytes. Previous studies on endothelial cells have demonstrated that regulated release of t-PA is dependent upon intracellular cAMP and Ca^{2+} levels[127]. Therefore, in the present study, forskolin was chosen as a direct activator of adenylate cyclase, and histamine as an activator of Ca^{2+}-dependent release. Following upregulation of t-PA by RA, a regulated release of t-PA from astrocytes was observed after 30 minutes of forskolin or histamine treatment (Figure 7). These results

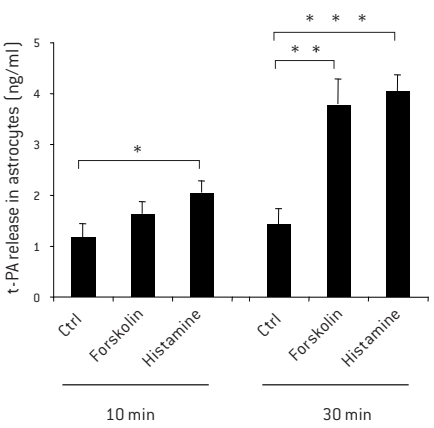


Figure 7. t-PA is released in a regulated manner from astrocytes. Cells were pre-treated with RA (10 µM) for 20 hours and exposed to forskolin (10 µM) or histamine (10 µM). Supernatants were collected after 10 or 30 minutes (min) to determine the regulated release of t-PA in response to each stimulus. Results are presented as the mean ± SEM. Each data-point represents the average of two treatment series, each performed on three separate occasions (n=6). Response to treatment was evaluated using an independent sample *t*-test; **P*<0.05, ***P*<0.01, ****P*<0.001, compared to the RA pre-treated control (Ctrl).

demonstrate the presence of astrocytic t-PA release pathways that involve both increased intracellular cAMP and Ca^{2+} levels.

What is the significance of an increased astrocytic t-PA expression in response to RA or PKC activating stimuli? During the embryonic development of the CNS, retinoids have been implicated in processes of neuronal proliferation, differentiation[167], and neurite outgrowth[168]. Retinoids are also generated within the adult hippocampus, and act to promote processes of learning and memory formation[169,170]. Activation of PKC has been associated with cerebral ischaemia[171], as well as with processes of neuronal regeneration and growth cone formation[172]. In light of our findings, we speculate that an increased astrocytic expression of t-PA is involved in a variety of physiological and pathophysiological processes in the developing brain, as well as in the adult brain.

Astrocytic expression of PAI-1 and PN-1 is regulated in a dynamic manner in response to injury-related factors (Paper II)

The importance of regulating the activity of serine proteases in the CNS is evident in numerous pathophysiological conditions, in which the balance between serine proteases and serpins may have an impact on clinical outcome. Therefore, the identification of factors that regulate serpin expression is important in terms of gaining further insight into the mechanisms that modulate proteolytic activities within the human brain. The experiments presented in Paper II were conducted to characterise the time-dependent effects of various injury-related factors on the regulation of PAI-1, PN-1, and neuroserpin in human astrocytes.

Several neuropathological conditions are associated with increased production of pro- and anti-inflammatory cytokines[173,174,175]. Therefore, the effects of various cytokines on PAI-1, PN-1, and neuroserpin expression were initially investigated. Early treatment (for 3 - 6 hours) with the pro-inflammatory cytokines interleukin 6 (IL-6) and TNF-α resulted in an approximate 2-fold upregulation of PAI-1 expression (mRNA and protein levels), which is in line with the results of previous studies on astrocytes of human origin[176,177]. Interestingly, prolonged exposure (14 - 20 hours) to these cytokines suppressed the expression of PAI-1. A similar response to interleukin 1β (IL-1β) has been described previously[176], although the mechanism underlying this inhibitory effect has not been determined. In addition, we observed that early exposure to the anti-inflammatory cytokine interleukin 10 (IL-10) induced an approximate 2-fold increase in the expression levels of PAI-1 and PN-1. Human astrocytes were also exposed to TGF-α and TGF-β, which are known inducers of PAI-1 expression in rodent astrocytes[89,178]. Early exposure resulted in a 2–3-fold increase in the astrocytic PAI-1 and PN-1 expression levels, whereas the expression

levels returned to the baseline levels after prolonged treatment with TGF-α and TGF-β. Hypoxia is also known to induce PAI-1 gene expression in various cell types[117], although the effect of this stimulus on astrocytic serpin expression has not been previously investigated. In a final set of experiments, we exposed astrocytes to an oxygen level of 1% and observed an approximate 2-fold upregulation of PAI-1 after 3 hours, whereas the expression level of PN-1 remained unchanged. It is noteworthy that the astrocytic PAI-1 response to hypoxia is modest compared to that reported for other cell types[179,180]. This may be attributed to species- or cell type-specific regulation of the PAI-1 gene[118], and/or variations in culturing conditions (e.g. number of cell passages, serum concentration in medium). The basal expression of neuroserpin in astrocytes was around the threshold of detection and showed no significant response to treatment. A schematic summary of the time-dependent mRNA expression patterns of PAI-1 and PN-1 observed following treatment with injury-related factors is given in Table 2.

Table 2. Time-dependent mRNA expression of PAI-1 and PN-1 in human astrocytes following treatment with injury-related factors.

Treatment	PAI-1		PN-1	
	Early (3–6 hrs)	Prolonged (14–20 hrs)	Early (3–6 hrs)	Prolonged (14–20 hrs)
Hypoxia	▲	+/-0	+/-0	+/-0
IL–6	▲	▲	▲	▲
IL–1β	▲	▼	▲	▲
TNF–α	▲	▼	▲	+/-0
IL–10	▲	+/-0	▲	+/-0
TGF–α	▲	+/-0	▲	+/-0
TGF–β	▲	+/-0	▲	+/-0

Dark arrows indicate strong upregulation or downregulation; light arrows indicate weak upregulation or downregulation; +/-0 indicates baseline expression levels (no significant effect of treatment).

Given that PAI-1 exerts neuroprotective effects[58, 84], induction of astrocytic PAI-1 expression might function as an endogenous mechanism to neutralise the deleterious effects of excessive serine protease activity following cerebral ischaemia. In support of this notion, immunohistochemical investigations of human brain tissue have demonstrated that PAI-1 expression is upregulated within the injured brain area following cerebral ischaemia[81, 82]. However, there is no apparent consistency in the literature as to how the expression of PAI-1 is affected by experimental cerebral ischaemia. These discrepancies probably reflect the different experimental models utilised (e.g. transient or permanent model), the duration of cerebral ischaemia, and/or species- or age-related differences.

Reactive astrocytes stain positively for t-PA and PAI-1 in human brain tissue (Papers I and II)

Reactive gliosis is a common feature of many neuropathologies. To explore the expression patterns of t-PA and PAI-1 in reactive astrocytes *in vivo*, we performed immunohistochemistry on brain biopsies obtained from patients undergoing surgery for intractable epilepsy. These experiments revealed strong and widespread immunoreactivities for t-PA and PAI-1 in reactive astrocytes as well as in cerebrovascular endothelial cells within gliotic brain areas. Experiments using double labelling with antibodies directed against the astrocyte intermediate filament protein GFAP showed that t-PA and PAI-1 are coexpressed with GFAP in the same astroglial cell. As cytoskeletal filaments have been implicated in the constitutive secretion of intracellular vesicles in glial cells[181], we speculated that GFAP plays a role in the storage and/or secretion of t-PA and/or PAI-1 in these cells.

Cocultivation of cerebrovascular endothelial cells and astrocytes potentiates astrocytic PAI-1 gene expression (Paper III)

Astrocytes are known to be regulators of brain endothelial functions[14]. Recent studies have indicated that astrocytes can affect fibrinolysis by enhancing the expression of PAI-1 while concurrently reducing the expression of t-PA in endothelial cells[182,183]. However, the regulatory effect of endothelial cells on the expression of these proteins in astrocytes has not been explored. Therefore, using a rodent *in vitro* model of the BBB, we investigated whether endothelial cells influence the expression levels of t-PA, PAI-1, and PN-1 in astrocytes.

When astrocytes were cocultured with cerebrovascular endothelial cells, the astrocytic PAI-1 mRNA expression levels were increased approximately

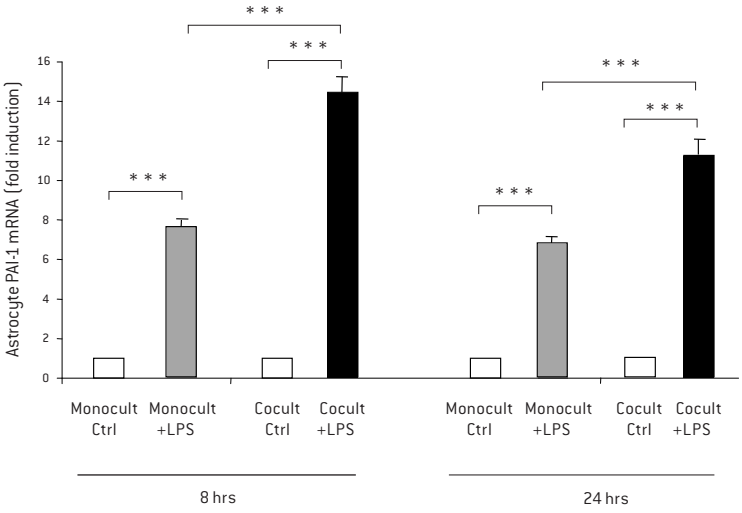


Figure 8. PAI-1 gene expression in astrocytes cocultivated with cerebrovascular endothelial cells is potentiated by LPS treatment. Monocultured or cocultured astrocytes were treated with LPS for 8 or 24 hours (hrs). In control cultures (untreated monocultured and cocultured cells), the medium was replaced with unsupplemented medium. Total cellular RNA was extracted, and mRNA was reverse-transcribed into cDNA. The levels of PAI-1 mRNA (cDNA) were quantified by qRT-PCR and normalised relative to the levels of GAPDH mRNA. Results shown are mean ± SEM fold-induction compared with the respective control culture. Three treatment series were performed on two separate occasions (n=6). Statistical analysis was performed using an independent sample t-test: ***P< 0.001.

5-fold, as compared to monocultured astrocytes. In contrast, no significant alterations in the expression levels of t-PA or PN-1 were observed. Lipopolysaccharide (LPS) is an endotoxin that is commonly used to induce inflammatory responses in experimental settings, and it is also a known stimulus for induction of PAI-1 gene expression[184,185]. In a second set of experiments, monocultures and cocultures of astrocytes and endothelial cells were treated with LPS for 8 or 24 hours. Pronounced increases in PAI-1 levels were observed after LPS treatment in both the monocultured and cocultured astrocytes, and induction was significantly higher in the cocultured astrocytes (Figure 8). LPS treatment did not induce any significant alterations in the expression levels of PN-1 or t-PA.

To investigate whether the LPS-mediated induction of PAI-1 in cocultured astrocytes is a persistent effect of endothelial-dependent conditioning or a direct effect of intercellular crosstalk, we performed an additional set of experiments in which the endothelial cells were removed from the cocultured astrocytes

before treatment with LPS. Even in the absence of endothelial cells, LPS treatment resulted in increased astrocytic expression of PAI-1, and this response was of similar magnitude as to that observed for astrocytes treated with LPS in the presence of endothelial cells. These findings imply an endothelial-dependent conditioning effect in astrocytes that remain after the endothelial cells are removed from the cocultures.

Although the mechanisms underlying the endothelial-dependent conditioning effect remain to be established, our data suggest that this is the result of astrocyte maturation and/or an increased secretion of astrocyte-derived factors, which may function in an autocrine fashion to induce PAI-1 gene expression. Both scenarios are plausible, since astrocytes mature during coculturing with endothelial cells[156, 186] and they express several cytokines that are potent inducers of PAI-1 gene expression, such as TGF-β[187], TNF-α, and IL-1β[188]. Recent data also demonstrate that LPS-induced release of IL-1β is enhanced in astrocytes that are cocultured with endothelial cells[186]. Therfore, we speculate that astrocyte-derived cytokines are involved in an autocrine signalling loop that is triggered by cocultivation with endothelial cells, and which can be further potentiated by LPS treatment. It is of note that, in contrast to the other studies presented in this thesis, the cells utilised in the *in vitro* BBB model were derived from rats. It would thus be of interest to investigate whether an endothelial-dependent conditioning effect of the same magnitude is generated in human astrocytes.

Allele-specific transcription of the PAI-1 gene in human astrocytes (Paper IV)

Several polymorphisms have been identified in the PAI-1 gene. Of these polymorphisms, the PAI-1 -675(4G/5G) promoter polymorphism is the most extensively studied. The 4G high-expressing allele has been associated with an increased risk of myocardial infarction[143,144], and with a reduced risk of ischaemic stroke[153,154]. One possible explanation for these apparently discrepant findings between myocardial infarction and stroke is that while PAI-1 can have an antifibrinolytic effect in both conditions, in stroke PAI-1 derived from astrocytes may have neuroprotective effects. The study presented in Paper IV was undertaken to investigate whether the PAI-1 -675(4G/5G) polymorphism affects PAI-1 gene expression in human astrocytes, using the novel haploChIP approach.

An initial critical step in these studies was to identify individuals who were heterozygous for the PAI-1 -675(4G/5G) polymorphism. Astrocytes derived from five individuals were cultured and genotyped by DNA sequencing, and

two individuals were found to be heterozygous for the PAI-1 -675(4G/5G) polymorphism. As any polymorphism located within 1 kb from the transcriptional start site can affect the haploChIP results, a 2-kb fragment surrounding the transcriptional start site was sequenced. The results demonstrated that the two individuals were also heterozygous for the PAI-1 -844A/G SNP, whereas no other additional heterozygosity was identified. As we had previously demonstrated that TGF-β is a potent stimulus for PAI-1 gene expression in human astrocytes (Paper II), the cells were pre-treated with TGF-β for 3 hours to induce PAI-1 gene transcription. Using the haploChIP approach, we observed a clear allele-specific difference in the transcriptional activity of the PAI-1 gene, as demonstrated by an increased recruitment of active RNA polymerase II to the haplotype that contained the 4G and the -844A alleles, as compared to the haplotype that contained the 5G and -844G alleles. Similar allele-specific effects were observed in untreated and in TGF-β treated cells.

To elucidate the molecular mechanisms behind these findings, EMSAs were performed. In the first set of EMSAs, probes that span the PAI-1 -675(4G/5G) site were used. These experiments revealed a specific binding of proteins when using nuclear extracts from human astrocytes. Two DNA-protein complexes were identified, one common band that bound with similar affinity to both alleles, whereas the second fast migrating band bound specifically to the 5G allele. We also observed similar DNA-protein migration patterns when using nuclear extracts from HUVECs, which is in accordance with previous findings[150]. However, in our hands, the 5G-specific complex was very weak and was only observed in approximately half of the EMSAs. Moreover, although previous studies have suggested that the 5G-specific complex contains a transcriptional repressor protein, attempts to identify this protein have been unsuccessful[149,150]. Therefore, additional supershift experiments were not performed in the present study.

As the PAI-1 -844A/G SNP may be of functional importance[141], DNA-protein interactions were also studied using probes that span the -844 site. These EMSAs confirmed the previous observation of the binding of a common protein complex to the G and A alleles. Additional supershift experiments identified the transcription factors IRF-2 and p53 as the major components of this complex. More importantly, we demonstrated for the first time an allele-specific binding of proteins to the -844G allele. *In silico* analysis of the -844 region predicted putative binding sites for factors belonging to the Ets-family of transcription factors. In agreement with these predictions, our supershift experiments revealed an -844G allele-specific binding of the transcription factors Elf-1 and Elk-1 (Figure 9).

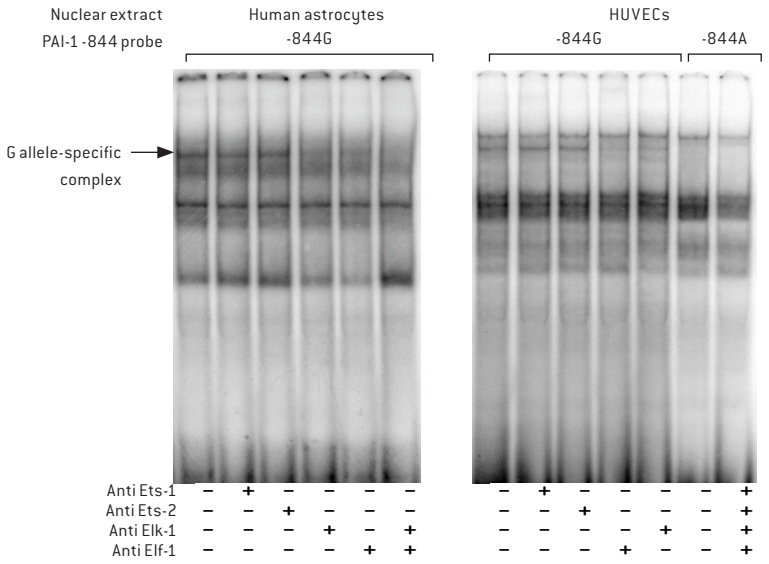


Figure 9. Elf-1 and Elk-1 bind to the PAI-1 -844G allele. Nuclear extracts from human astrocytes (A) or HUVECs (B) were incubated with ^{32}P-labelled G or A probes with or without the addition of antibodies directed against Ets-1, Ets-2, Elk-1 or Elf-1 as indicated.

Our results demonstrate that genetic variants within the PAI-1 promoter affect the transcriptional activity of the PAI-1 gene in human astrocytes, and suggest that both the PAI-1 -675(4G/5G) and the -844A/G polymorphisms are of functional importance in these cells. Given the potential neuroprotective effect of PAI-1, we propose that an increased astrocytic expression of PAI-1, as conferred by genetic PAI-1 promoter variants, reduces brain damage following an ischaemic event. If this is indeed the case, then our findings provide a possible molecular genetic mechanism for the association between PAI-1 promoter variants and ischaemic stroke.

The t-PA -7351C>T enhancer SNP affects t-PA gene expression in human brain tissue (Paper V)

Our group has previously demonstrated that the t-PA -7351C>T enhancer SNP is functional at the level of transcription and affects t-PA gene expression in endothelial cells *in vitro*[139,140], as well as endothelial t-PA release *in vivo*[138]. Given the multifaceted role of t-PA under physiological and pathophysiolgical conditions in the brain, we addressed the question as to whether the t-PA -7351C>T SNP affects the expression of the t-PA gene in human brain tissue.

The t-PA -7351C>T SNP is in LD with the coding t-PA 20099T>C SNP, with a D' of 0.93[138,189], which allowed us to use this coding SNP as a marker for the allele-specific gene expression analysis. Post-mortem brain tissue samples were obtained from 42 donors. Twelve of these donors were identified as being heterozygous for both SNPs, and samples from these individuals were thus selected for the subsequent analysis. Using the quantitative TaqMan genotyping assay, we found that the wild-type C allele produced approximately 20% and 30% more t-PA mRNA transcripts than the mutant T allele in the cortical and hippocampal brain tissue samples, respectively ($P<0.05$ for both). It is noteworthy that not all the individuals showed an allele-specific gene expression of t-PA (Figure 10). These differences may be explained by inter-individual and region-specific variations in the expression levels of the t-PA gene. This notion is supported by our previous observation that the effect of the t-PA -7351C>T SNP is more pronounced under conditions of enhanced t-PA gene expression[138,139,140].

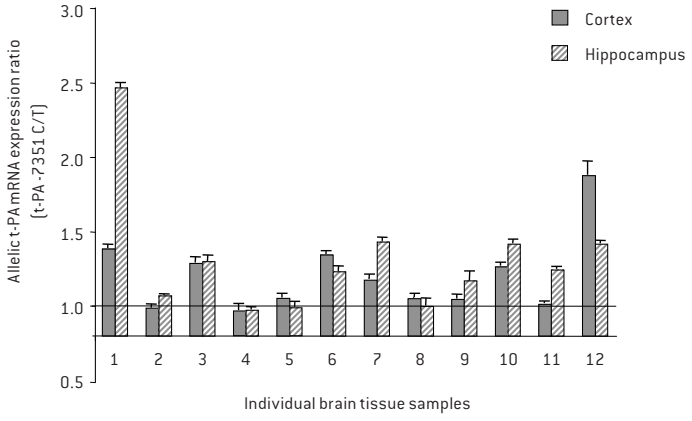


Figure 10. Individual allelic ratios of t-PA gene expression in human brain tissue. Cortical and hippocampal samples (n=24) heterozygous for the coding t-PA 20099T>C SNP and the t-PA -7351C>T SNP were investigated using the quantitative TaqMan genotyping assay. The horizontal line represents a ratio of 1, assuming equivalent expression of each allele. Each bar represents the cDNA allelic ratio for each individual brain tissue sample. All samples were assayed in three separate reactions, and each experiment was repeated on two separate occasions.

To determine whether the t-PA -7351C>T SNP affects binding of transcription factors in brain-derived cells, EMSA experiments were performed. Our results demonstrated a substantially reduced nuclear protein binding affinity for the T allele probe, as compared to that for the C allele counterpart, when using nuclear extracts from human astrocytes or NT2 cells. Cross-competition experiments revealed that the protein binding affinity for the C allele was at least 10-fold greater than for the T allele, which is in line with our previous observations for endothelial cells[139]. As this polymorphic site has previously been shown to bind Sp1 and Sp3 in nuclear extracts from HUVECs[139], antibodies directed against the Sp1-family of transcription factors were included in the additional supershift experiments. These experiments demonstrated that Sp1 and Sp3 are the two major transcription factors that bind to the polymorphic site in human astrocytes and NT2 cells.

The present findings suggest that the t-PA -7351C>T enhancer SNP is a determinant for t-PA gene expression in human brain tissue. With regard to the biological significance of this finding, it is feasible to speculate that if the wild-type C allele confers an increased t-PA gene expression, then this allele is potentially beneficial for learning and memory formation and for functional recovery after cerebral ischaemia. On the other hand, although a high intravascular expression of t-PA is likely to have profibrinolytic effects and thus reduce the risk of thrombotic occlusion in the acute phase of cerebral ischaemia, high-level expression of t-PA within the brain parenchyma may have deleterious effects as this could trigger proteolytic cascades and make neurons more vulnerable to excitotoxic damage. Therefore, although we have not detected any significant difference in allelic frequency between ischaemic stroke patients and controls[190], the t-PA -7351C>T SNP may have an impact on the final infarct size following cerebral ischaemia and/or for stroke recovery.

CONCLUSIONS TO GIVEN AIMS

In human astrocytes, RA and the PKC activatior PMA are potent stimuli for the induction of t-PA gene expression. t-PA is stored in intracellular granules and released in a regulated manner from these cells. These findings may be important for elucidating the role of astrocyte-derived t-PA.

The expression levels of PAI-1 and PN-1 in human astrocytes are regulated in a dynamic manner by injury-related factors, which may form part of the defence mechanism to protect the brain tissue from excessive serine protease activity.

Cocultivation of astrocytes and cerebrovascular endothelial cells potentiates the expression of PAI-1 in astrocytes. These findings underline the importance of intercellular crosstalk in the modulation of astrocytic expression of PAI-1 within the BBB.

The PAI-1 -675(4G/5G) and the -844A/G polymorphisms affect the transcriptional activity of the PAI-1 gene in human astrocytes. This finding provides a potential molecular genetic mechanism behind the association between PAI-1 promoter variants and ischaemic stroke.

The t-PA -7351C>T SNP affects t-PA gene expression in human brain tissue. This finding may have clinical implications for neurological conditions associated with enhanced t-PA levels, such as in the acute phase of cerebral ischaemia, and for stroke recovery.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

There is an increasing body of evidence demonstrating that the actions of t-PA within the brain are highly pleiotropic, as a result of its interplay with several extracellular molecules, receptors, and inhibitors. As new molecular targets and novel functions of t-PA and its inhibitors are continuously being discovered, it is clear that additional studies are required to unravel the specific roles of these proteins in the normal and diseased brain.

Our observations that the astrocytic expression profiles of t-PA and its inhibitors PAI-1 and PN-1 are regulated in a dynamic manner by various physiological and pathophysiological stimuli imply that these cells are important modulators of extracellular t-PA activity in the CNS. We also demonstrate that the intercellular signalling between astrocytes and cerebrovascular endothelial cells is of importance for astrocytic control of extracellular t-PA activity at the neurovascular interface. In light of our findings, it is interesting to note that there is evidence to suggest that t-PA exerts different effects dependent upon its cellular, temporal, and spatial expression patterns. This is exemplified by experiments conducted by Tsirka and co-workers, in which they have demonstrated that excitotoxin-induced secretion of t-PA by neurons acts initially as a signal to activate microglial cells, whereas the subsequent secretion of t-PA by microglial cells promotes ECM degradation and neurodegeneration[191]. Interestingly, a more recent study demonstrated that astrocytic expression of t-PA induced by treatment with multipotent mesenchymal stromal cells is beneficial for regenerative processes, in that it promotes neurite outgrowth following cerebral ischaemia[192]. Taken together, these findings underscore the complex roles of t-PA within the brain.

The fact that both t-PA and its inhibitors are regulated in a dynamic and complex manner makes studies on the pathopysiological role of the plasminogen activation system in the human brain very challenging. For natural reasons, it is not feasible to investigate the expression of these proteins in the brain in different clinical settings. An alternative approach is to investigate regulatory polymorphisms, i.e., genetic variants that affect gene expression. Such polymorphisms could be used as markers of inter-individual differences in the expression levels of specific genes. In this context, the present thesis provides evidence that both the t-PA and PAI-1 genes contain regulatory polymorphisms that affect the expression levels of the respective genes in human brain tissue. These polymorphisms could thus be used as informatic tools to investigate the significance of differences in the expression

levels of t-PA and PAI-1 in adequately powered clinical case-control studies on neurological conditions, such as cerebral ischaemia, neurodegenerative diseases, and epilepsy. It would also be of interest to evaluate the potential of these genetic markers as providers of prognostic information in longitudinal studies of patients with these neurological conditions.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och hjärninfarkt uppstår vanligen till följd av att en blodpropp bildas i något av de kärl som försörjer dessa livsviktiga organ. Härigenom förhindras blodflödet helt eller delvis, ofta med allvarliga konsekvenser. Då en blodpropp bildas aktiveras ett inneboende försvarssystem mot blodproppsbildning, vilket bland annat resulterar i att ett blodproppsupplösande ämne frisätts till blodbanan. Detta ämne kallas för vävnadsplasminogenaktivator (t-PA) och frisätts från det lager av celler som täcker blodkärlets inre vägg, så kallade endotelceller. I blodet finns också ämnen som kan hämma och därigenom balansera t-PAs proppupplösande förmåga, och den viktigaste hämmaren är plasminogenaktivatorinhibitor typ 1 (PAI-1).

Senare års forskning har visat att t-PA även produceras i hjärnan, där dess roll har visat sig vara betydligt mer komplex än i blodet. Bland annat är t-PA involverad i utvecklingen av nervsystemet under fosterlivet, medan t-PA hos vuxna har betydelse för bland annat minne och inlärning. I motsats till dessa gynnsamma effekter har t-PA även visat sig kunna bidra till de skadliga processer som sker i hjärnvävnaden vid en infarkt eller hjärnskada. Mot denna bakgrund var syftet med den aktuella avhandlingen att undersöka hur produktionen av t-PA och PAI-1 regleras, med fokus på den mänskliga hjärnan.

Delarbete I visar för första gången att den vanligaste celltypen i hjärnan, så kallade astrocyter, kan lagra och frisätta t-PA som svar på yttre stimuli. Dessa fynd tyder på att astrocyter kan bidra till ökade nivåer av t-PA i hjärnan. Eftersom astrocyter anses vara den huvudsakliga källan till PAI-1 i hjärnan syftade delarbete II till att undersöka hur produktionen av PAI-1, samt ytterligare en hämmare till t-PA, protease nexin (PN-1), påverkas av skaderelaterade faktorer så som syrebrist och inflammation. Resultaten visar att produktionen av PAI-1 och PN-1 ökar då cellerna exponeras för syrebrist eller inflammatoriska mediatorer. Detta svar kan utgöra en viktig skyddsmekanism mot skadliga effekter orsakade av t-PA i hjärnan. Ett exempel på t-PAs ogynnsamma effekter är dess förmåga att bryta ner den barriär som skyddar hjärnan från främmande ämnen, den så kallade blod-hjärnbarriären. Delarbete III syftade därför till att studera hur produktionen av t-PA, PAI-1 och PN-1 regleras i astrocyter då dessa celler odlas tillsammans med endotelceller i en experimentell modell som kan liknas vid blod-hjärnbarriären. Dessa experiment visade att endotelceller kan öka produktionen av PAI-1 i astrocyter och att denna effekt förstärks vid inflammation. Vår grupp har tidigare visat att en vanligt förekommande variation i t-PA genen

påverkar produktionen av t-PA i endotelet. Våra samarbetspartners har gjort motsvarande fynd för PAI-1. I delarbete IV och V visar vi att dessa genetiska varianter påverkar produktionen av t-PA och PAI-1 även i hjärnan, och beskriver även möjliga mekanismer för hur detta sker.

Sammanfattningsvis bidrar dessa resultat till en fördjupad kunskap kring hur t-PA och PAI-1 regleras i hjärnan. På längre sikt kan den förvärvade kunskapen öka vår förståelse kring hur t-PA påverkar minne och inlärningsprocesser samt olika sjukdomstillstånd som förknippas med förändrade nivåer av t-PA i hjärnan, såsom hjärninfarkt och demens.

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